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An Equine Herpesvirus Type 1 Recombinant with a Deletion in the gE and gI Genes Is Avirulent in Young Horses

Tomio Matsumura,[1] Takashi Kondo, Shigeo Sugita, Armando M. Damiani,*
Dennis J. O'Callaghan,† and Hiroshi Imagawa

*Epizootic Research Station, Equine Research Institute, Japan Racing Association, Kokubunji-machi, Shimotsuga-gun, Tochigi 329-04, Japan; *Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yayoi, Bunkyou-ku, Tokyo 113, Japan; and †Department of Microbiology and Immunology, Louisiana State University Medical Center, 1501 Kings Highway, P.O. Box 33932, Shreveport, Louisiana 71130*

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The cell culture-adapted KyA strain of equine herpesvirus type 1 (EHV-1) has been found to be attenuated in young horses (Matsumura *et al.,* 1996, *Vet. Microbiol.* 48, 353–365). The KyA strain lacks at least six genes in its genome, including those encoding glycoproteins gE and gI. To elucidate whether EHV-1 glycoproteins gE and gI play a role in viral virulence, we have constructed an EHV-1 recombinant that has the genes encoding both gE and gI deleted from its genome and its revertant. Growth properties of the deletion mutant virus *in vitro* were compared with those of the parent and the revertant viruses. Plaque size of the mutant virus in fetal horse kidney (FHK) cells was significantly smaller than those of the parent and the revertant viruses. In one-step growth experiments, however, the yields of infectious virus from FHK cells infected with the deletion mutant, the parent, or the revertant virus were approximately the same. The results suggested that gE and/or gI of EHV-1 promoted cell-to-cell spread of the virus, but that these glycoproteins were not involved in the process of virus maturation and release or in virus attachment and penetration. Subsequently, the virulence of mutant and revertant viruses was examined in young horses. No clinical signs were observed in six horses, including three colostrum-deprived foals inoculated intranasally with the deletion mutant virus, whereas three colostrum-deprived foals inoculated intranasally with the revertant virus manifested clinical signs typical for EHV-1 respiratory infection (i.e., pyrexia, nasal discharge, and swelling of submandibular lymph nodes). The results obtained from *in vivo* studies revealed that the EHV-1 mutant defective in both gE and gI genes was avirulent in young horses, suggesting that gE and/or gI of the EHV-1 have an important role in EHV-1 virulence. However, the EHV-1 mutant defective in both gE and gI genes induced only a partial protectivity in inoculated foals from manifestation of respiratory symptoms after challenge infection.

INTRODUCTION

Equine herpesvirus type 1 (EHV-1), a member of the Alphaherpesvirus subfamily, causes abortion and nervous disorder in susceptible mares and epizootic respiratory disease in racehorses (Allen and Bryans, 1986; Mason *et al.,* 1989; Matsumura *et al.,* 1992; O'Callaghan and Osterrieder, 1998). When widely distributed, EHV-1 causes serious economic loss in the horse industry worldwide. Although vaccines against EHV-1 are available, their efficacy is limited (Burrows *et al.,* 1984; Bürki *et al.,* 1990). Progress in genetic engineering technology has allowed the construction of Alphaherpesviruses that are attenuated due to the deletion of DNA sequences essential for viral virulence (Kimman *et al.,* 1992; van Engelenburg *et al.,* 1994; Kaashoek *et al.,* 1996; Kruger *et al.,* 1996; Yokoyama *et al.,* 1996). Such recombinant viruses would be candidates for development of an effective live vaccine that could stimulate both antibody and cellular immune responses important in the protection against herpesvirus infection (Rouse and Horohov, 1984). A vaccine against EHV-1, in addition, should be completely safe, since it would be inoculated into racehorses, which are delicate animals to maintain in excellent condition for races. For development of an effective and safe live EHV-1 vaccine, it is important to determine the functions necessary for viral pathogenicity. However, knowledge concerning the genetic basis for the virulence of EHV-1 is limited. Among the 76 genes deduced from the complete DNA sequence of the EHV-1 Ab4p strain (Telford *et al.,* 1992), genes 67 (IR6) and 71 (EUS4) have been associated with the virulence of EHV-1 for mice (Osterrieder *et al.,* 1996; Marshal *et al.,* 1997). To date, gene 38, which encodes thymidine kinase (TK), is the only gene confirmed associated with the virulence of EHV-1 for the natural host, horses (Cornick *et al.,* 1990; Slater *et al.,* 1993), although an EHV-1 TK-deficient mutant still exhibits mild virulence for specific pathogen-free (SPF) foals (Slater *et al.,* 1993).

Recent studies revealed that a laboratory strain of EHV-1, Kentucky A (KyA), has no virulence for horses

[1] To whom correspondence and reprint requests should be addressed. Fax: 81-285-40-1064. E-mail: tomats@epizoo.equinst.go.jp.

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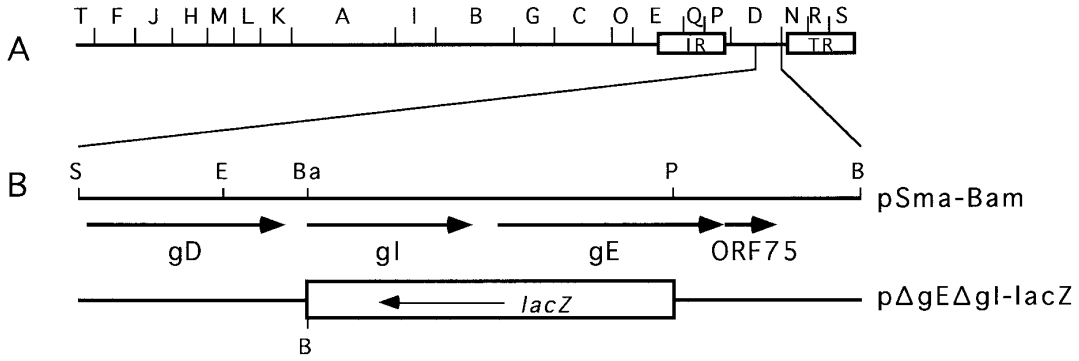

FIG. 1 Diagram of the EHV-1 genome. (A) The *Bam*HI restriction map of the EHV-1 HH-1 strain (Kirisawa *et al.,* 1993b). (B) The 5.8-kb *Sma*I–*Bam*HI fragment cloned into pUC vector (pSma-Bam) is expanded, showing relevant restriction sites (B, *Bam*HI; Ba, *Bal*I; E, *Eco*RI; P, *Pma*CI; S, *Sma*I). The locations and direction of ORF 75 and genes encoding gD, gI, and gE are indicated with arrows. Shown below is the construction of deletion–insertion vector (pΔgEΔgI-lacZ) derived from pSma-Bam, in which the location and direction of *lacZ* gene insertion are indicated. By the insertion, a *Bam*HI recognition site is newly generated in pΔgEΔgI-lacZ.

(Matsumura *et al.,* 1996) or for mice (Colle *et al.,* 1996). Due to extensive passage in nonequine cells (Flowers and O'Callaghan, 1992) and in hamsters (Randall and Lawson, 1962), the KyA strain has lost at least six genes (genes 1, 2, 17, 73, 74, and 75) compared with the genomic sequence of the EHV-1 Ab4p strain (Yalamanchili and O'Callaghan, 1990; Yalamanchili *et al.,* 1990; Flowers and O'Callaghan, 1992; Telford *et al.,* 1992; Matsumura *et al.,* 1993). Gene 17, a homolog of varicella zoster virus (VZV) gene 15, encodes a particularly hydrophobic protein containing a periodic charge pattern (Karlin *et al.,* 1989; Telford *et al.,* 1992). Gene 1 has a homolog in pseudorabies virus (PRV) (Baumeister *et al.,* 1995), and gene 75 has positional homologs in herpes simplex virus type 1 (HSV-1) (Georgopoulou *et al.,* 1993) and feline herpesvirus type 1 (FHV-1) (Willemse *et al.,* 1995), although no definite functions of proteins encoded by genes 1 and 75 as well as gene 2 have yet been elucidated. On the other hand, genes 73 and 74 encode glycoproteins gI and gE, respectively (Audonnet *et al.,* 1990; Elton *et al.,* 1991; Telford *et al.,* 1992), homologs of which are associated with viral virulence in Alphaherpesviruses. HSV-1 mutants unable to express gE or gI failed to induce corneal disease or encephalitis in mice following corneal inoculation (Dingwell *et al.,* 1994). Glycoproteins gI and gp63 of PRV, homologs of gE and gI of HSV-1, respectively, play a role in the invasion and spread of virus in the nervous system of pigs (Kritas *et al.,* 1994). Furthermore, deletion of the gE gene of BHV-1 or the gE and gI genes of FHV-1 reduced viral virulence in the natural hosts, bovine and feline, respectively (van Engelenburg *et al.,* 1994; Sussman *et al.,* 1995; Kruger *et al.,* 1996). Thus, we have expected that the lack of gE and gI genes in the KyA genome would contribute to an attenuated nature of the KyA strain.

In this paper, we describe the construction of a gE and gI deletion mutant from a virulent EHV-1 strain and its revertant and report the features of the mutant and revertant viruses exhibited *in vitro* and *in vivo. In vitro* studies suggested that the gE and/or gI of EHV-1 facilitated cell-to-cell virus spread. Furthermore, *in vivo* studies revealed that the gE and gI deletion mutant of EHV-1 was avirulent for horses and that the revertant virus rescued the inherent virulence of EHV-1 for foals.

RESULTS

Construction of a gE and gI deletion mutant and its revertant

To construct a deletion–insertion vector, plasmid pSma-Bam containing EHV-1 genes for gD, gI, gE, and 75 was digested with *Bal*I and *Pma*CI to remove 2747 nucleotides (nt 132,902 to 135,648 of EHV-1 Ab4p strain; GenBank Accession No. M86664) that contain most of the coding sequences for the gI and gE genes (Fig. 1). The flanking sequence was ligated with the blunt-ended 4.2-kb DNA fragment, containing the *E. coli lacZ* gene and the SV40 early promoter, which was obtained by digestion of pCH110 with *Tth*111I and *Bam*HI. The resulting plasmid, designated pΔgEΔgI-lacZ, lacks 1271 of 1275 bp and 1244 of 1653 bp of the EHV-1 gI and gE sequences, respectively. Insertion of *lacZ* gene in the opposite direction to the gD, gI, gE, and 75 genes of EHV-1 resulted in the generation of a new *Bam*HI recognition site at the junction site of the *Bal*I end of EHV-1 and the *Bam*HI end of pCH110 (Fig. 1B). An EHV-1 virus stock containing the *lacZ*-positive mutant was obtained by transfection of pΔgEΔgI-lacZ DNA into FHK cells infected with the 89c25p strain. Purification of the EHV-1 mutant was achieved after four repetitive procedures of blue plaque cloning, and the resulting mutant was designated ΔgEΔgI-lacZ. Construction and purification of a revertant virus were similarly achieved by transfecting pSma-Bam DNA into FHK cells infected with the ΔgEΔgI-lacZ strain and by repetitive procedures of white plaque

TABLE 1

Nucleotide Sequences of Primers for PCR and Sequencing

Primers	Sequence	Location[a]
EHV-1		
Sma sequence up	5′-GCTTGCCCCACGAATCTAGT	131,001–131,020
Sma sequence down	5′-CAAGCAGCACAGCAGGCATA	131,450–131,431
Deletion sequence up	5′-ATCAGCGTCGGTTTGGGTATCG	132,635–132,656
Deletion sequence down	5′-TCATACGGCTTACGGTTGCTTC	135,723–135,702
Bam sequence up	5′-CGACCAAGAAGTAAGCGAAG	136,859–136,878
Bam sequence down	5′-CGACGACACAACCACCATCT	137,120–137,101
gI up	5′-GAATCATGCCCGCGTGTAGCCA	133,174–133,195
gI down	5′-GTGCAGGCGATGTGGTACGAAG	133,768–133,747
gE up	5′-AGTCACATATCGTAGAGCCCAG	135,072–135,093
gE down	5′-CTACAGATGGATGAGGAGTGAT	135,581–135,560
gB FC2[b]	5′-CTTGTGAGATCTAACCGCAC	62,908–62,927
gB RC (reverse)[b]	5′-GGGTATAGAGCTTTCATGGG	64,088–64,069
gB FC3[b]	5′-ATACGATCACATCCAATCCC	63,180–63,199
gB R1 (reverse)[b]	5′-GCGTTATAGCTATCACGTCC	63,367–63,348
pCH110		
lacZ-Bam sequence	5′-GCTATTTCTCTGTTCTCGCTAT	3544–3565
lacZ-Tth sequence	5′-GTGAAAACCTCTGACACATGCA	6758–6737
lacZ-1 up	5′-GACCAGAAACAGCACCTCGAAC	220–241
lacZ-1 down	5′-GGTTTTCTCCGGCGCGTAAAAA	817–796
lacZ-3 up	5′-ACATCCCCCTTTCGCCAGCT	357–376
lacZ-3 down	5′-TGCACCACAGATGAAACGCCG	733–713

[a] Nucleotide numbers of EHV-1 and pCH110 primers correspond to the complete sequences of EHV-1 Ab4p strain (GenBank Accession No. M86664) and pCH110 (GenBank Accession No. U13845), respectively.

[b] Primers for nested PCR to detect EHV-1 gB gene were synthesized according to the published sequences described by Kirisawa *et al.* (1993a).

cloning, respectively. The resulting revertant was designated gE-gI-rev.

By PCR using gE, gI, and lacZ-1 primer sets (Table 1), only the *lacZ* product was amplified from ΔgEΔgI-lacZ DNA, while the gE and gI products but no *lacZ* product were amplified from gE-gI-rev DNA. DNA sequence analyses using sequence primers listed in Table 1 revealed that the pCH110-derived sequence was inserted in the appropriate location on the ΔgEΔgI-lacZ genome and that no alteration was observed in the EHV-1 sequences around the junction sites (*Sma*I, *Bal*I(*Bam*HI), *Pma*CI, and *Bam*HI sites; see Fig. 1) of the ΔgEΔgI-lacZ compared to the corresponding sequences of the parent 89c25p strain and the Ab4p strain of EHV-1 (Telford *et al.,* 1992). In Southern blot analysis on *Bam*HI digests of viral DNAs, furthermore, the pSma-Bam DNA probe hybridized to two fragments of ΔgEΔgI-lacZ DNA, a 6.4- and a 5.6-kb fragment, as expected from the newly generated *Bam*HI recognition site by the insertion of the pCH110-derived sequence and to a single 10.5-kb *Bam*HI D fragment of 89c25p and gE-gI-rev DNAs (Fig. 2). DIG-labeled pCH110 DNA failed to hybridize to any fragment of 89c25p and gE-gI-rev DNAs, but hybridized to the 5.6-kb fragment of ΔgEΔgI-lacZ DNA (data not shown). These results confirmed that the ΔgEΔgI-lacZ genome lacked the 2747 nucleotides from the fifth nucleotide of the gI open reading frame (ORF) to nucleotide 1244 of the gE ORF, including the entire noncoding sequence (232 nt) located between the gI and gE ORFs, and that the deleted EHV-1 sequence in the ΔgEΔgI-lacZ genome was rescued in the gE-gI-rev genome.

Growth properties of EHV-1 strains *in vitro*

In experiments concerning viral growth, the KyA strain of EHV-1 was used as a reference strain, as the KyA genome lacks gE and gI genes by a 3859-bp deletion from nt 132,811 to 136,669 relative to the sequence of

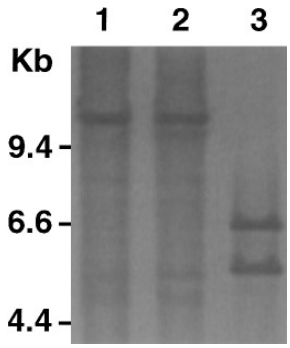

FIG. 2. Southern blot analysis of EHV-1 DNAs digested with *Bam*HI and probed with pSma-Bam (Fig. 1). Digested DNAs were electrophoresed through 0.7% agarose gels for 18.5 h at 20 V, stained with ethidium bromide, photographed, and then transferred to nylon membrane. Lane 1, gE-gI-rev DNA; lane 2, 89c25p DNA; and lane 3, ΔgEΔgI-lacZ DNA. The sizes of molecular markers are indicated on the left.

TABLE 2

Plaque Size of EHV-1 Strains in FHK Cells

Strain	Days after inoculation	
	3	5
89c25p	1.92 ± 0.20[a]	3.72 ± 0.22
gE-gI-rev	1.86 ± 0.18	3.60 ± 0.20
ΔgEΔgI-lacZ	0.71 ± 0.12	1.45 ± 0.23
KyA	1.25 ± 0.17	2.36 ± 0.24

[a] Mean ± standard deviation ($n = 20$) of plaque size (mm).

EHV-1 Ab4p strain (Flowers and O'Callaghan, 1992; Telford *et al.*, 1992). Since the gE and gI genes of other herpesviruses have been associated with the cell-to-cell spread of virus, the plaque size of the ΔgEΔgI-lacZ was compared to that of the parent 89c25p, the revertant gE-gI-rev, and the KyA strains in FHK cells. The average sizes of 20 plaques formed after 3 and 5 days cultivation of the strains lacking gE and gI genes, ΔgEΔgI-lacZ and KyA, were significantly smaller ($P < 0.0001$) than those of the intact EHV-1 strains, 89c25p and gE-gI-rev (Table 2). Moreover, a significant difference in the average sizes of plaques was also observed between the deletion strains, ΔgEΔgI-lacZ and KyA ($P < 0.0001$). No difference was observed statistically between the plaque sizes of 89c25p and gE-gI-rev strains. These results suggested that the gE and/or gI play an important role in cell-to-cell viral spread for EHV-1 as shown for other herpesviruses.

Next, a one-step growth experiment of EHV-1 strains was conducted to examine whether the deletion of the gE-gI sequences would affect viral growth in FHK and/or RK13 cells. Cells were infected and harvested at 0, 6, 12, 24, 36, 48, and 72 h postinoculation. Infectivity titers of extracellular and intracellular viruses at each time were determined by the plaque titration using FHK cells. The virus titers of extracellular and intracellular samples from FHK cells inoculated with ΔgEΔgI-lacZ, 89c25p, or gE-gI-rev strains reached the peak titers at 48 and 36 h postinoculation, respectively (Fig. 3). The growth patterns of these three strains were quite similar, although the virus titer of the ΔgEΔgI-lacZ extracellular sample at 12 h postinoculation was higher to some degree than those of the parent and revertant strains. In contrast, the growth curves of extracellular and intracellular KyA showed a rapid increase and reached peak levels at 24 h postinoculation, and the extracellular and intracellular titers of KyA showed the highest value at each time point among the four strains examined. The results obtained by using RK13 cells were almost identical to those obtained by using FHK cells (data not shown). The finding that the ΔgEΔgI-lacZ virus could replicate to almost the same level as the parent strain in cell cultures con-

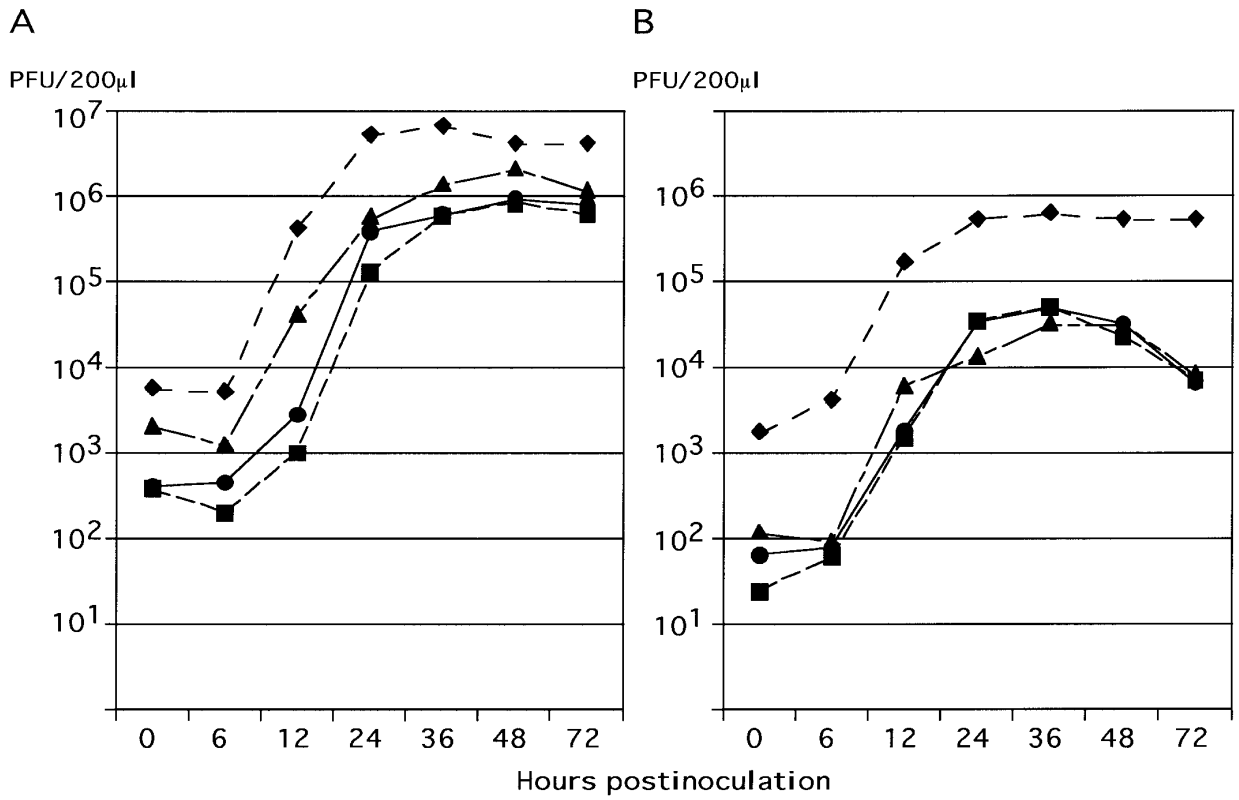

FIG. 3. One-step growth curves of EHV-1 strains in FHK cells. (A) Extracellular and (B) intracellular virus titers (PFU/200 μl). (■) 89c25p; (●) gE-gI-rev; (▲) ΔgEΔgI-lacZ; and (◆) KyA strains.

TABLE 3

Duration of Clinical Symptoms Observed and of Virus Isolation in Horses after Inoculation with EHV-1 Strains ΔgEΔgI-lacZ and/or gE-gI-rev

Symptoms and results of virus isolation	Post ΔgEΔgI-lacZ inoculation (EI-1–EI-6)	Post gE-gI-rev inoculation	
		ΔgEΔgI-lacZ-inoculated foals (EI-4–EI-6)	Control foals (R-1–R-3)
Pyrexia (≧39°C)	Negative	3 to 8 (5.7 ± 2.5)[a] days	4 to 7 (5.3 ± 1.5) days
Nasal discharge	Negative	0[b] to 7 (3.0 ± 3.6) days	3 to 7 (5.0 ± 2.0) days
Swelling of L.N.[c]	Negative	0[d] or 8 days	10 to 15 (13.0 ± 2.6) days
Virus shedding	0 or 1 day[e]	7 to 8 (7.7 ± 0.6) days	6 to 8 (7.0 ± 1.0) days
Viremia	Negative	7 to 8 (7.3 ± 0.6) days	8 to 10 (9.3 ± 1.2) days

[a] Mean ± standard deviation shown in parentheses.
[b] Nasal discharge was not recorded in a foal (EI-4).
[c] Submandibular lymph nodes.
[d] Swelling of the lymph nodes was not recorded in two foals (EI-4 and EI-5).
[e] *LacZ*-positive virus was isolated only from a horse (EI-1) on day 2 postinoculation.

firmed that the gE and the gI of EHV-1 are not required for viral growth *in vitro.* The remarkable growth property of the KyA strain *in vitro* may be due to some other modifications apart from the deletion of the gE and gI genes on the KyA genome, which has been acquired during a long history of passage in nonequine cells.

Virulence of the deletion mutant and the revertant

Inoculations of the ΔgEΔgI-lacZ ($10^{7.0}$ PFU/horse) and the gE-gI-rev strain ($5 \times 10^{6.0}$ PFU/foal) were given intranasally to six young horses including three colostrum-deprived foals (EI-1–EI-6) and three colostrum-deprived foals (R-1–R-3), respectively. All horses inoculated with the ΔgEΔgI-lacZ showed no clinical symptoms of respiratory disease (Table 3). In contrast, an obvious elevation in rectal temperature (≧39.0°C) was observed for 4 to 7 days from day 1 or 2 postinoculation in the three foals (R-1–R-3) inoculated with the gE-gI-rev strain (Table 3; indicated as control foals). Furthermore, serous to mucopurulent nasal discharge was observed for 5, 3, and 7 days from day 4 postinoculation, and swelling of submandibular lymph nodes was observed for 15, 10, and 14 days from days 3, 5, and 4 postinoculation in foals R-1, R-2, and R-3, respectively. Clinical signs observed in the foals inoculated with the gE-gI-rev strain of EHV-1 corresponded well to those in the weanling horses inoculated with the 89c25 strain of EHV-1 (Matsumura *et al.,* 1996). These findings indicated that the gE-gI deletion mutant of EHV-1, ΔgEΔgI-lacZ, was attenuated and that the revertant EHV-1 derived from the ΔgEΔgI-lacZ exhibited the inherent virulence of EHV-1 to cause a respiratory disease in foals.

Clinical signs of ΔgEΔgI-lacZ inoculated foals after challenge

Based on the result that the gE-gI-rev retained virulence for foals, the gE-gI-rev strain of EHV-1 was used as a challenge virus to examine the protection of the colostrum-deprived foals (EI-4–EI-6) at 4 weeks after inoculation with the ΔgEΔgI-lacZ strain. The results obtained from the experimental gE-gI-rev infection in the three foals were used as control data for this challenge experiment (R-1, R-2, and R-3 were indicated as control foals in Tables 3–5). After the intranasal challenge with the gE-gI-rev ($5 \times 10^{6.0}$ PFU/foal), serous to mucopurulent nasal discharge was observed for 7 and 2 days from days 2 and 3 postchallenge in foals EI-5 and EI-6, respectively, and swelling of submandibular lymph nodes was observed for 8 days from day 5 postchallenge in foal EI-6 (Table 3). These clinical signs were not observed in one foal, EI-4. However, an obvious elevation in rectal temperature was observed in all foals for 3 to 8 days from day 1 or 2 postchallenge. These results suggested that only a partial protection against challenge with pathogenic EHV-1 was conferred on foals by intranasal inoculation of the ΔgEΔgI-lacZ.

Virus isolation

After the inoculation with the ΔgEΔgI-lacZ strain, a mutant strain of EHV-1 confirmed by the *lacZ* gene expression and PCR was isolated only from a nasal swab collected from one (EI-1) of the six horses on day 2 postinoculation (Table 3). No other EHV-1 strain was isolated even from autopsied samples. These findings indicated that intranasal inoculation with the gE and gI deletion mutant of EHV-1 did not result in significant virus shedding. In additional assays to detect the gE-gI deletion mutant DNA, approximately 10 mg of minced trigeminal ganglion, thymus, and lymph nodes samples [10 autopsied samples from each of the horses (EI-1–EI-3)] were subjected to DNA extraction, and the resulting samples were examined by nested PCR using the published primer sets to detect the EHV-1 gB DNA (Kirisawa *et al.,* 1993a) and lacZ-1 and lacZ-3 primer sets to detect

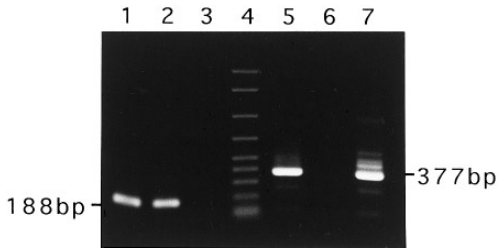

FIG. 4. Ethidium bromide-stained agarose gel of representative nested PCR products. Lanes 1–3, EHV-1 gB-specific primer pairs (see Table 1) used for the nested PCR; lane 4, DNA size standard, 50- to 2000-bp ladder (Bio-Rad Laboratories, CA); lanes 5–7, *E. coli LacZ*-specific primer pairs used. The following templates were employed: Lane 1, EHV-1 89c25p DNA; lanes 2 and 5, DNA sample extracted from submandibular lymph nodes of horse EI-3; lanes 3 and 6, DNA sample extracted from the trigeminal ganglion of horse EI-3; lane 7, EHV-1 ΔgEΔgI-lacZ DNA.

the *lacZ* DNA (Table 1). Both 188 bp of the gB sequence of EHV-1 and 377 bp of the *lacZ* sequence were amplified only from DNA extracted from submandibular lymph nodes of horse EI-3 by nested PCR (Fig. 4), and no amplification of the gB- and/or the *lacZ*-specific sequences was detected from the other 29 samples, including the trigeminal ganglion samples as well as from a negative control water. On the other hand, the gB and the *lacZ* sequences were amplified from the control DNAs of the 89c25p and ΔgEΔgI-lacZ strains, respectively (Fig. 4). The gB sequence was also amplified from ΔgEΔgI-lacZ DNA, but no *lacZ* product was amplified from 89c25p DNA by nested PCR (data not shown). After challenge with the gE-gI-rev strain, on the other hand, EHV-1 strains were isolated consecutively for 7 to 8 days from day 1 and for 7 to 8 days from days 2–4 postchallenge from nasal and blood samples, respectively (Table 4). These findings closely resembled the results of virus isolation from gE-gI-rev-inoculated control foals (R-1–R-3), as shown in Tables 3 and 4. The results suggested that prior intranasal inoculation of foals with the ΔgEΔgI-lacZ strain had little effect in reducing the duration of virus shedding and viremia after infection with the virulent EHV-1.

Antibody responses of horses

Antibody responses of horses to EHV-1 detected by serum neutralization (SN) and CF tests are shown in Table 5. SN antibody to EHV-4 was not detected in the serum of any horses throughout the study. None of the six horses inoculated with the ΔgEΔgI-lacZ strain exhibited SN antibody response to EHV-1, and two of them exhibited a weak CF antibody response at a 3-week point (EI-3) and from 3 weeks (EI-1) after the inoculation of ΔgEΔgI-lacZ strain. On the other hand, two of the three foals inoculated with the gE-gI-rev strain exhibited SN antibody response to EHV-1 from 2 (R-3) and 3 weeks (R-1) after the inoculation, and CF antibody responses were recorded in all of these foals from 2 weeks after the inoculation of gE-gI-rev strain. After the challenge with the gE-gI-rev strain, SN antibody responses of the foals inoculated 4 weeks prior with the ΔgEΔgI-lacZ strain were similar to those of the control foals (R-1–R-3). However, CF antibody responses observed in the ΔgEΔgI-lacZ-inoculated foals after challenge were detected one week earlier and were higher in titer compared to those of the control foals. The results indicated that an anamnestic boost in CF antibody response, but not in SN antibody response, did occur following challenge with a virulent EHV-1 in foals immunized intranasally with the ΔgEΔgI-lacZ strain.

DISCUSSION

We describe the construction of a recombinant EHV-1 with a deletion in the gE and gI genes and its revertant and some of the biological characteristics of these viruses identified in *in vitro* and *in vivo* studies. Insertion of the *lacZ* gene of *E. coli* replaced almost the entire coding

TABLE 4

EHV-1 Isolation from Foals after Inoculation with gE-gI-rev Strain

	Days after inoculation of gE-gI-rev strain											
Foal	0	1	2	3	4	5	6	7	8	10	12	14–21
ΔgEΔgI-lacZ-inoculated foals												
EI-4	—	N[a]	NM[b]	NM	NM	NM	NM	NM	NM	—	—	—
EI-5	—	N	N	NM	NM	NM	NM	NM	M	M	—	—
EI-6	—	N	N	N	NM	NM	NM	NM	NM	M	—	—
Control foals												
R-1	—	NM	NM	NM	NM	NM	NM	NM	M	M	—	—
R-2	—	NM	N	NM	N	NM	NM	NM	NM	M	—	—
R-3	—	N	N	NM	NM	NM	NM	M	M	M	M	—

[a] EHV-1 was isolated from a nasal swab.
[b] EHV-1 was isolated from a peripheral monocyte fraction.

TABLE 5

Antibody Titers against EHV-1 Detected in Nine Horses Inoculated with EHV-1 Strains ΔgEΔgI-lacZ and/or gE-gI-rev

Horse	Test[a]	Antibody titers at indicated weeks after inoculation of ΔgEΔgI-lacZ strain								
		0	1	2	3	4[b](0)	5(1)	6(2)	7(3)	8(4)
ΔgEΔgI-lacZ-inoculated horses										
EI-1	SN	<1:5	<1:5	<1:5	<1:5	<1:5				
	CF	<1:4	<1:4	<1:4	1:4	1:4				
EI-2	SN	<1:5	<1:5	<1:5	<1:5	<1:5				
	CF	<1:4	<1:4	<1:4	<1:4	<1:4				
EI-3	SN	<1:5	<1:5	<1:5	<1:5	<1:5				
	CF	<1:4	<1:4	<1:4	1:4	<1:4				
EI-4	SN	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	1:5	1:5	ND[c]
	CF	<1:4	<1:4	<1:4	<1:4	<1:4	1:32	1:128	1:128	ND
EI-5	SN	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	1:5	1:5	1:5
	CF	<1:4	<1:4	<1:4	<1:4	<1:4	1:32	≥1:256	1:128	1:128
EI-6	SN	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	1:5	<1:5
	CF	<1:4	<1:4	<1:4	<1:4	<1:4	1:16	≥1:256	1:128	1:128
Control foals										
R-1	SN					<1:5	<1:5	<1:5	1:5	1:5
	CF					<1:4	<1:4	1:32	1:64	1:32
R-2	SN					<1:5	<1:5	<1:5	<1:5	<1:5
	CF					<1:4	<1:4	1:32	1:32	1:32
R-3	SN					<1:5	<1:5	1:10	1:10	ND
	CF					<1:4	<1:4	1:32	1:32	ND

[a] Serum neutralization (SN) tests were conducted using EHV-1 89c25 strain. Complement fixation (CF) tests were conducted using an antigen prepared from the EHV-1 HH-1 strain.

[b] Challenge of EHV-1 gE-gI-rev strain was conducted in 3 ΔgEΔgI-lacZ-inoculated foals (EI-4 to EI-6) and 3 control foals (R-1 to R-3). Weeks after challenge of gE-gI-rev strain are represented in parentheses.

[c] Not done.

sequence for the gI gene, the promoter for the gE gene, and most of the gE 5′ sequence. Subsequently, the inserted *lacZ* gene was replaced by the deleted EHV-1 sequence in the recombinant. The resulting deletion virus, ΔgEΔgI-lacZ, and its revertant, gE-gI-rev, were verified by PCR, DNA sequencing, and Southern blot analyses. Serial passage of EHV-1 in nonequine cell cultures has been shown to result in alterations in the viral DNA (Allen *et al.,* 1983). As expression of the *lacZ* gene was hardly detected in viable FHK cells in the presence of X-gal, nonequine RK13 cells were used for the selection of the mutant virus. However, no alteration in the EHV-1 genome except for that engineered in the gI/gE locus of the Us segment was detected in the RE profiles and DNA sequencing of the viral strains used in this study.

The functions of the gE and the gI of other herpesviruses have been assessed using recombinant viruses lacking either or both of these glycoprotein genes. Glycoproteins gE and gI of herpesviruses have been shown to facilitate cell-to-cell spread since the deleted viruses yield small plaques compared to those of the parent viruses in the case of HSV-1 (Neidhardt *et al.,* 1987; Dingwell *et al.,* 1994), PRV (Jacobs *et al.,* 1993), BHV-1 (Otsuka and Xuan, 1996), and FHV-1 (Sussman *et al.,* 1995). The fact that the ΔgEΔgI-lacZ strain of EHV-1 produced significantly smaller plaques in FHK cells than did the parent virus suggested that gE and/or gI of the EHV-1 also facilitate cell-to-cell spread of EHV-1. Furthermore, the time course of replication of the ΔgEΔgI-lacZ strain in FHK and RK13 cells was quite similar to that of the parent strain, notwithstanding the low cell-to-cell infectivity, suggesting that gE and gI of the EHV-1 are not involved in either the process of virus maturation and release or virus attachment and penetration. Compared to the virulent EHV-1, the KyA strain of EHV-1, which lacks at least six genes including gE and gI, also produced small plaques which were, however, larger than those of the ΔgEΔgI-lacZ strain. Moreover, the KyA strain replicated to titers that exceeded those of the virulent and the gE and gI deletion mutant strains of EHV-1. Since the complete sequencing of the KyA strain is not yet accomplished, the other modifications in the KyA genome apart from the 3859-bp deletion, including the coding sequences for gE and gI, may account for its plaque size and its efficient growth in cell culture.

A PRV gE/gI deletion mutant derived from a virulent PRV (Ka) strain exhibited reduced virulence, but still possessed the ability to kill young pigs following intranasal inoculation (Mettenleiter *et al.,* 1987). Similarly, mild clinical signs were observed in cats inoculated oronasally with the recombinant FHV-1, in which the genes for gE and gI were deleted (Kruger *et al.,* 1996).

Therefore, the findings that horses inoculated intranasally with a high dose of the recombinant EHV-1 strain, ΔgEΔgI-lacZ, exhibited no clinical signs were unexpected (Table 3). Furthermore, since the gE-gI revertant virus caused respiratory symptoms typical of EHV-1 infection in foals, it would appear that gE and gI may be important factors in EHV-1 virulence. These findings also suggest that the attenuated nature of the KyA strain may have resulted from the deletion of gE and gI genes (Flowers and O'Callaghan, 1992).

After the inoculation of six horses with the ΔgEΔgI-lacZ strain, virus replication of the ΔgEΔgI-lacZ strain in the upper respiratory tract was detected in only one (EI-1) animal (Table 3). However, amplification of the DNA sequences of the ΔgEΔgI-lacZ strain was confirmed in submandibular lymph nodes collected from one (EI-3) of the three horses autopsied 4 weeks after the ΔgEΔgI-lacZ inoculation. EHV-1 has been found to establish latency in lymphoid tissues draining the respiratory tract (Welch *et al.,* 1992), and recent reports indicated that EHV-1 establishes latency within the trigeminal neurons (Slater *et al.,* 1994; Baxi *et al.,* 1995). Although limited replication in the respiratory tract and no viremia were observed in horses inoculated with the ΔgEΔgI-lacZ strain, the ΔgEΔgI-lacZ strain may possess the ability to establish latency or to persist in lymph nodes. Although sequences of neither the EHV-1 gB gene nor the *lacZ* gene were amplified from trigeminal ganglia of three horses inoculated with the ΔgEΔgI-lacZ strain, further investigation will be necessary to ascertain whether the gE/gI deletion virus can replicate and establish latency in the central nervous system of the equine.

The antibody responses against EHV-1 were weak or undetectable in six horses after inoculation with the ΔgEΔgI-lacZ virus. However, an anamnestic boost in CF antibody response in three ΔgEΔgI-lacZ-inoculated foals (Table 5) and protection from manifestation of nasal discharge and swelling of submandibular lymph nodes in one (EI-4) and two (EI-4 and EI-5) of the three foals, respectively (Table 3), were observed after the challenge. On the other hand, the SN antibody response to EHV-1 and the duration of the virus isolation from both nasal swab and peripheral blood samples of the ΔgEΔgI-lacZ-inoculated foals after the challenge were similar to those observed in the control foals (Tables 4 and 5). In our previous study (Matsumura *et al.,* 1996), duration of virus shedding and viremia were shortened remarkably and a higher and earlier SN antibody response to EHV-1 was observed in the KyA-inoculated horses after the challenge with the pathogenic EHV-1 89c25 strain compared to those of the control horses. The results of the present study indicated that abilities of ΔgEΔgI-lacZ to stimulate the humoral antibody response and to confer protectivity against the virulent EHV-1 infection in intranasally inoculated horses were rather weak compared to those of the virulent and the KyA strains. In this regard, a similar SN antibody response was observed in cats inoculated oronasally with the recombinant FHV-1 defective in gE and gI genes, in which no SN antibody response was recorded on day 21 postinoculation (Kruger *et al.,* 1996). However, subcutaneous administration of the recombinant FHV-1 induced SN antibody titers of cats to a level as high as that of virulent FHV-1 on day 21 postinoculation. Similarly, intramuscular inoculation with BHV-1 mutants induced good SN antibody titers in cattle compared to intranasal inoculation (Liang *et al.,* 1997). Although natural postnatal EHV-1 infection occurs via the respiratory route, different routes of inoculation may increase the immunogenicity of the recombinant EHV-1 defective in the gE and gI genes in horses.

The results from this study demonstrate that the gE and gI genes of EHV-1 are important virulence factors and that an EHV-1 mutant defective in these genes is safe if inoculated intranasally in horses. Since the gE and gI genes of other herpesviruses are associated with neurovirulence (Neidhardt *et al.,* 1987; Card *et al.,* 1992; Kritas *et al.,* 1994) and since EHV-1 can cause nervous disorders in infected horses, deletion of the nonessential gE and gI genes may be important in developing safe recombinant EHV-1 vaccines and EHV-1-based vaccine vectors. To elucidate the functions of the gE and gI genes of EHV-1 more precisely, construction of EHV-1 recombinants deleted with either of gE and gI genes is under way.

MATERIALS AND METHODS

Viruses, cells, and media

The EHV-1 strain 89c25p, a plaque-purified strain of a low-passaged field strain, 89c25, was used as a parent virus to construct the EHV-1 mutant. The strain, 89c25, was isolated from a racehorse involved in the epizootic of EHV-1 respiratory infection in 1989; during this epizootic some of the infected horses manifested nervous disorders (Matsumura *et al.,* 1992, 1994). The 89c25 strain induces respiratory infection in yearlings (Matsumura *et al.,* 1996), and fetal infection results from experimental infection of pregnant mares (unpublished data). Neither the plaque-purified nor the original 89c25 strain has been passaged in nonequine cells. The EHV-1 89c25 strain and an EHV-4 strain, TH20, the Japanese prototype of EHV-4 (Kawakami *et al.,* 1962), were used to detect neutralizing antibodies to EHV-1 and EHV-4, respectively, in the sera of experimentally infected horses. For a preparation of complement fixation (CF) antigen, EHV-1 strain HH-1, the Japanese prototype of EHV-1 (Kawakami *et al.,* 1970), was used. An EHV-1 laboratory strain, KyA, was also used as a gI- and gE-negative reference strain of EHV-1 (Flowers and O'Callaghan, 1992).

Fetal horse kidney (FHK, within the seventh passage history from primary culture) cells were used throughout

the study except for selection of a mutant virus, for which RK13 (an established cell line of rabbit kidney) cells were used. FHK and RK13 cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Both FHK and RK13 cells were used for one-step growth experiments. RK13 and MDBK (an established cell line of bovine kidney) cells grown in EMEM supplemented with 10% calf serum and antibiotics were used for virus isolation from samples taken from experimentally infected horses.

Plasmids and cloning

Molecular cloning procedures were essentially as described by Sambrook *et al.* (1989). The viral DNA of EHV-1 89c25p strain was extracted from infected FHK cells as described previously (Matsumura *et al.,* 1996). Digestion of 89c25p DNA with *Sma*I and *Bam*HI yielded a 5.8-kb fragment, deduced from the complete DNA sequence of EHV-1 Ab4p (Telford *et al.,* 1992) to include genes gI and gE. The 5.8-kb fragment was cloned into the *Hin*cII and *Bam*HI sites of plasmid pUC19, and the recombinant plasmid was designated as pSma-Bam (Fig. 1). Plasmid pSma-Bam was confirmed to contain the EHV-1 fragment corresponding to the *Sma*I–*Bam*HI (nucleotides (nt) 131,238 to 137,006) fragment of EHV-1 Ab4p DNA by restriction enzyme (RE) and DNA sequence analyses. Plasmid pCH110 containing the *Escherichia coli lacZ* gene under the SV40 early promoter control was purchased from Pharmacia Inc. (NJ).

Homologous recombination and recombinant screening

Linearized donor plasmid DNA (20 μg) was mixed with FHK cell suspensions [2×10^6 cells/0.8 ml phosphate-buffered saline (PBS)] infected 1 h previously with the 89c25p strain at a m.o.i. of 0.02, and the mixture in a 0.4-cm cuvette was electroporated (0.3 kV, 250 μF) using gene transfer equipment (Gene Pulser, Bio-Rad Laboratories, CA). Transfected cells were grown in 6-well culture plates at 37°C in a 5% CO_2 atmosphere and were harvested with a cell scraper at 4 days when cytopathic effects (CPE) appeared. Cells were disrupted by three freeze–thaw cycles and cleared by centrifugation. To confirm whether the resulting virus stocks contained a mutant virus expressing the *lacZ* gene, serially diluted virus stocks were inoculated onto FHK monolayer in 6-well plates, and cells were cultured for 3 days with EMEM containing 0.75% methylcellulose and 5% FCS. The cells were then fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Takara Biomedicals, Japan) by the method of Sanes *et al.* (1986) with a slight modification. After one wash with PBS, cells were fixed with 2% (v/v) formaldehyde and 0.2% glutaraldehyde solution in PBS for 5 min at room temperature, washed twice with PBS, and incubated with a staining solution consisting of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml of X-gal in PBS at 37°C for 3 h. The cells that stained blue were regarded as positive for the expression of *lacZ* gene.

Selection of the mutant virus expressing the *lacZ* gene was conducted by seeding appropriately diluted virus stocks on RK13 cells in 90-mm dishes. After 3 days incubation at 37°C with the overlay EMEM containing 5% FCS and 0.9% agar, the cells were further overlaid with the 0.9% agar medium containing 0.5 mg/ml of X-gal and incubated at 37°C for 24 to 48 h. Blue plaques containing recombinants were isolated and inoculated again onto RK13 cells. This blue plaque cloning procedure was repeated until 100% of the plaques in fixed cells were positive for X-gal staining. Subsequently, purified virus was passaged on FHK cells to yield virus stocks for further experiments. Similarly, a revertant virus generated by homologous recombination between the mutant virus and plasmid pSma-Bam DNA was selected by white plaque cloning on RK13 cells under the presence of X-gal in the overlay medium.

Polymerase chain reaction (PCR) and DNA sequencing

Primers listed in Table 1 were used in PCR and DNA sequencing to identify the deletion of gI and gE sequences and the insertion of *lacZ* gene in a deletion–insertion mutant and to confirm the stability of the EHV-1 sequences at the junction sites (*Sma*I and *Bam*HI sites) of homologous recombination within the mutant virus genome. The primers were synthesized with the Applied Biosystems DNA synthesizer (Model 392). In addition, nested PCR was conducted to detect the viral DNA in some autopsied samples collected from experimentally infected horses. PCR was carried out in a volume of 50 μl containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each dNTP, 1.0 μM each primer, an appropriate amount of DNA sample, and 1.25 units of *Taq* polymerase (Perkin–Elmer, ABI, Japan). DNA amplification was performed for 35 cycles, each consisting of denaturation at 94°C for 75 s, annealing at 60°C for 90 s, and extension at 72°C for 90 s. Sequencing reactions were carried out with cycle sequencing kits (Perkin–Elmer, ABI, Japan), and the labeled DNA was sequenced with the Applied Biosystems DNA Sequencer (Model 373A).

Southern blot hybridization

Digoxigenin (DIG)-labeled pSma-Bam and pCH110 DNAs were used as probes for hybridization. Viral DNA fragments digested with *Bam*HI were separated on agarose gels by electrophoresis and transferred to nylon membranes as described by Sambrook *et al.* (1989). Hybridization assays and chemiluminescent

detection of the probed fragment(s) were performed using the DIG luminescent detection kit under conditions recommended by the manufacturer (Boehringer Mannheim, FRG).

Plaque size measurement

Appropriately diluted viruses were inoculated onto FHK monolayers in 35-mm dishes (Corning Inc., Corning, NY). Following 1 h adsorption, cells were overlaid with EMEM containing 0.75% methylcellulose and cultured for 3 and 5 days. Cells were then fixed and stained with 0.2% crystal violet or 1 mg/ml X-gal. Plaque-size measurements were performed using the Electronic Plate Reader AD001 (Alta Diagnostic Machines Ltd., Birmingham, England). The Student's *t* test was employed to compare the means of plaque sizes ($n = 20$) of examined viruses.

Time course of viral growth

Monolayers of FHK and RK13 cells prepared in 6-well plates were inoculated with virus samples at a m.o.i. of 4 plaque-forming unit (PFU)/cell. After 1 h adsorption, cells were washed three times with EMEM and incubated at 37°C in a 5% CO_2 atmosphere in 2 ml/well of EMEM with 2% FCS. At various intervals after inoculation, culture fluids with scraped cells were centrifuged to sediment the infected cells. The supernatants were used as the extracellular samples. Following two washes with EMEM, the cell pellets were resuspended in 2 ml of EMEM with 2% FCS and subjected to three freeze–thaw cycles. After centrifugation, the resulting supernatants were used as the intracellular samples. Both extracellular and intracellular samples were titrated for viral infectivity.

Animal experiments

To assess the virulence of the viruses, six young horses [three half-bred weanling horses aged 9 months (designated EI-1, EI-2, and EI-3) and three colostrum-deprived Thoroughbred foals aged 71–85 days (designated EI-4, EI-5, and EI-6)] were inoculated intranasally with the deletion mutant, and three colostrum-deprived Thoroughbred foals aged 68–77 days (designated R-1, R-2, and R-3) were inoculated intranasally with the revertant. By confirmation that the revertant was as virulent as the parent EHV-1 (see Results), three foals inoculated with deletion mutant virus (EI-4–EI-6) were challenged with the revertant 4 weeks later. All foals (EI-4–EI-6 and R-1–R-3) were apart from their dams for 24 h after birth, during which time the foals were fed a commercial milk substitute and the dams' udders were regularly stripped out to remove the immunoglobulin-rich colostrum, as described by Jeffcot (1973). All horses were free of neutralizing antibodies against EHV-1 and EHV-4, which are antigenically cross-reactive and cross-protective (Allen and Bryans, 1986). Rectal temperatures were recorded twice a day, and clinical signs were recorded at the time of sample collection. Three conventionally reared horses (EI-1–EI-3) infected with the deletion mutant virus were autopsied on days 27–28 postinoculation to detect the mutant virus distribution. For virus isolation, nasal swab and peripheral blood samples were collected daily for the first week after the inoculation or the challenge, every other day for the second week, and twice a week thereafter from each horse. Samples collected at autopsy were as described previously (Matsumura *et al.,* 1996). Serum samples were collected at weekly intervals and stored at −20°C until use.

Virus isolation

Virus isolation was conducted immediately after the sample collection. Preparations of inocula from collected samples for virus isolation were described previously (Matsumura *et al.,* 1996). Approximately 10^6 mononuclear cells fractionated using Lymphoprep (Nycomed, Oslo, Norway), 0.5 ml of nasal and autopsied fluid samples, and approximately 10 mg of minced tissue samples were cocultured with suspended cells in 25-cm^2 flasks (Corning Inc., Corning, NY). Flasks were incubated at 37°C and observed for CPE daily for a week.

Assay for serum antibody to EHV-1 and EHV-4

For detection of type-specific antibody to EHV-1 and EHV-4, plaque neutralization tests were conducted as described previously (Matsumura *et al.,* 1996). CF assays were also carried out to detect antibody reactive with both EHV-1 and EHV-4, as described by Sugiura *et al.* (1987).

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